

The invention features novel peptide derivatives called Reversins, and provides for their use in a method of reducing the activity of the multi-drug transporter protein MDR1 in order to overcome multidrug resistance in a mammal. The peptide derivatives are of the formula (I) $X^1_n-X^2-X^3(X^4)_n-X^5$, wherein n is 0 or 1, and each n is the same or different; X^1 is BOC, BOC-Asu, Z-Asu, benzyloxycarbonyl, Glu(OBzl)-OBzl, Trp-OMe, Trp-Phe-OMe, Phe-Trp-OMe, Phe-Phe-OtBu, Trp-Trp-OtBu, indoloacetyl, benzoyl, an alkylamine of 1-4 carbons, dibenzylamide, tryptamide, 1-amino-adamantane, aminomethylcyclohexane, indoline, phenylethylamide or dicyclohexylamide; X^2 is Glu(OBzl), Asp(OBzl), succinyl, O,O-dibenzoyltartaroyl, diphenoyl, muconyl, Thx, Cpa, Asu, Nal, Pen, Phg, Dbt, Lys(BOC), Lys(Z), Cys(Bzl), Thr(Bzl), Glu(OtBu), *tert*-Leu, Leu, Nle, Pro, Phe, Tyr(Bzl), or Ser(Bal); X^3 is Asp, Asu, Lys, Glu, Trp, Thx, Cpa, Nal, Pen, Phg, Dbt, Glu(OtBu), *tert*-Leu, Leu, Nle, Pro, Tyr, Phe, or Tyr(Bzl); X^4 is BOC-Glu(OBzl), Glu(OBzl), Asu, OBzl, Bzl, BOC, BOC-Lys(BOC), Z-Glu(OtBu), Asp(OBzl), Asp(OBz)-OBzl, benzyloxycarbonyl, O-(cyclo-hexyl), fluorenylmethyl ester, Glu(OtBu), Glu(OtBu)-OBzl, 1-amino-adamantane, aminomethylcyclohexane, indoline, phenylethylamide, or dicyclohexylamide; and X^5 is OMe, OBzl, OtBu, Phe-OMe, O-(cyclohexyl), Trp-OMe, (chlorophenyl)-isobutylamide, fluorenylmethyl ester, ONp, 1-aminoadamantane, aminomethylcyclohexane, indoline, phenylethylamide, or dicyclohexylamide.

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COMPOUNDS FOR REVERSING DRUG RESISTANCEBackground of the Invention

5 This invention relates to compounds for overcoming resistance that a patient may build to therapeutics.

Treatment of many diseases can be severely limited by resistance to the chosen therapeutic drug. For example, chemotherapy, while generally an effective
10 treatment against human cancerous diseases, is hampered when a patient becomes resistant to the chemotherapeutic. In one special form of drug resistance, called "Multidrug Resistance," the cell becomes resistant not only to the chemotherapeutic being administered, but to a wide range
15 of structurally and functionally unrelated drugs simultaneously (see Ford et al., *Pharmacological Reviews*, 42:155-199, 1992).

The cause of multidrug resistance is the appearance of an integral glycoprotein in the plasma
20 membrane of the targeted cell, e.g., a tumor cell (Fig. 1). The protein functions as a multidrug transporter, and is variously called MultiDrug-Resistance 1 protein (MDR1), P-glycoprotein (pleiotropic-glycoprotein), Pgp, or P-170. MDR1 consists of 1280 amino acid residues, and
25 contains 12 transmembrane segments and two nucleotide-binding domains. It strongly resembles prokaryotic and eukaryotic members of the so-called ABC (ATP Binding Cassette) transporters, or traffic ATPases (see Endicott et al., *Annu. Rev. Biochem.* 58:137-171, 1989; Higgins,
30 *Annu. Rev. Cell. Biol.* 8:67-113, 1992).

MDR1 naturally functions to, and is highly expressed in tissues normally responsible for, extruding toxic materials and waste-products from cells (e.g., lung, kidney, and liver), and secretes hydrophobic
35 compounds from exocrine or endocrine glands (Gottesman et

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al., *J. Biol. Chem.* 263:12163-12166, 1988; Higgins et al., *supra*). Consistent with its natural function, MDR1 catalyses an ATP-dependent extrusion of various cytotoxic drugs from the cell, e.g., vinca alkaloids, anthracyclines, and other natural antibiotics, thereby maintaining their cellular level at a subtoxic concentration. Thus, when expressed by tumor cells, MDR1 expels cytotoxic chemotherapeutic agents, and thus allows the tumor cell to survive anticancer treatments even at high drug doses. At the same time, "ordinary" cells, having no such extrusion mechanism, may receive a lethal drug exposure. Tumors developing from tissues normally expressing the MDR1 protein often show a primary drug resistance, while in other tumors a secondary drug resistance may develop after chemotherapy.

The phenomenon of multidrug resistance is not limited to tumor cells. MDR1 and its homologues are expressed in a wide variety of cell-types, including parasitic protozoa. Consequently, overexpression of a member of the MDR1 family of proteins creates obstacles to a wide variety of parasitic diseases, including malaria, African sleeping sickness, and others (Campbell et al., *Chemotherapy of Parasitic Diseases*, Plenum Press:NY, 1986; Henderson et al., *Mol. Cell. Biol.* 12:2855-65, 1992). MDR1 is also expressed by endothelial cells of human capillary blood vessels at the blood-brain barrier and blood-testis barrier (Ford et al. *supra*, at 159).

It is known that verapamil, a drug that blocks voltage-dependent calcium channels, stimulates the activity of MDR1-bound ATPase at a concentration of 1 to 20 μ M but inhibits it as a concentration above 100 μ M (Sarkadi et al. *J. Biol. Chem.* 267:4854-4858, 1992). While between these concentrations verapamil blocks the extrusion of antitumor drugs, its high toxicity severely

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limits its clinical use (Solary et al. *Leukemia* 5:592-597, 1991; Dalton et al. *J. Clin. Oncology* 7:415-418, 1989).

Summary of the Invention

5 The invention generally features chemical compositions which reduce or overcome multidrug resistance in a mammal, e.g., a human, and in microorganisms causing disease in a mammal. The compounds, called "Formula (I)" compounds, or

10 "Reversins", are hydrophobic peptide derivatives which effectively compete with cytostatic drugs on the MDR1 protein, thus reducing or eliminating drug resistance.

"Multi-drug resistance", as used herein, refers to the ability of cells to develop resistance to a broad

15 range of structurally or functionally unrelated drugs. This occurs by outward transport of the drug from the cell, the transport being mediated by the MDR1 glycoprotein or its homologues. The term "multidrug resistance" also applies to the cross-resistance between

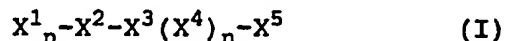
20 drugs which is adversely affected by the Reversin compounds of the invention (see below). Preferably, "multidrug resistance" refers to the state which is dependant on expression or overexpression of the MDR1 protein or its homologues, and/or on gene amplification

25 of human mdr1 or its homologues. Both primary and secondary multidrug resistance are included. Where the drug resistance is "primary" the cell has experienced no previous exposure to a member of the group of drugs, yet exhibits inherent resistance to them. Where drug

30 resistance is "secondary", the cell has been exposed to only one drug, or to only a subset of two or more, but not necessarily to the whole, group of drugs affected by cross-resistance.

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The compounds of the invention, hereafter called "Reversins", are of formula (I):



wherein

- 5 n is 0 or 1, and each n is the same or different;
 X¹ is BOC, BOC-Asu, Z-Asu, benzyloxycarbonyl, Glu(OBzl)-OBzl, Trp-OMe, Trp-Phe-OMe, Phe-Trp-OMe, Phe-Phe-OtBu, Trp-Trp-OtBu, indoloacetyl, benzoyl, an alkylamine of 1-4 carbons, dibenzylamide, tryptamide, 1-amino-adamantine, aminomethylcyclohexane, indoline, phenylethylamide or dicyclohexylamide;
 10 X² is Glu(OBzl), Asp(OBzl), succinyl, O,O-dibenzoyltartaroyl, diphenoyl, muconyl, Thx, Cpa, Asu, Nal, Pen, Phg, Dbt, Lys(BOC), Lys(Z), Cys(Bzl), Thr(Bzl), Glu(OtBu), tert.-Leu, Leu, Nle, Pro, Phe, Tyr(Bzl), or Ser(Bzl);
 15 X³ is Asp, Asu, Lys, Glu, Trp, Thx, Cpa, Nal, Pen, Phg, Dbt, Glu(OtBu), tert.-Leu, Leu, Nle, Pro, Tyr, Phe, or Tyr(Bzl);
 20 X⁴ is BOC-Glu(OBzl), Glu(OBzl), Asu, OBzl, Bzl, BOC, BOC-Lys(BOC), Z-Glu(OtBu), Asp(OBzl), Asp(OBz)-OBzl, benzyloxycarbonyl, 0-(cyclo-hexyl), fluorenylmethyl ester, Glu(OtBu), Glu(OtBu)-OBzl, 25 1-amino-adamantine, aminomethylcyclohexane, indoline, phenylethylamide, or dicyclohexylamide; and
 X⁵ is OMe, OBzl, OtBu, Phe-OMe, -O-(cyclohexyl), Trp-OMe, (chlorophenyl)-isobutylamide, fluorenylmethyl ester, ONp, 1-aminoadamantane, aminomethylcyclohexane, indoline, phenylethylamide, or dicyclohexylamide.
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Formula (I) compounds containing amino acids with either the L or D configuration fall within the scope of the invention.

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Side chain protecting groups of amino acids may be substituted by one or more halogen atoms, e.g., chloro-Z, or bromo-Z. Such blocking groups are known. A benzyl ester group can be substituted by one or more nitro groups in the second or fourth position of the benzene ring.

The abbreviations used herein are known to those skilled in the art (see, e.g., *J. Biol. Chem.* 241:527, 1966; *J. Biol. Chem.* 247:977 1972; hereby incorporated by reference). Other abbreviations used herein are as follows:

	AM:	acetoxymethyl ester
	Asu:	aminosuccinic acid or aminosuccinoyl
	BOC:	<u>tert.</u> -butyloxycarbonyl,
15	Bzl:	benzyl,
	Cpa:	4-chlorophenylalanyl,
	Cys:	cysteinyl,
	Dbt:	dibromotyrosyl,
	DBTA:	dibenzoyltartaroyl,
20	DCC:	dicyclohexylcarbodiimide,
	DCU:	dicyclohexylurea,
	DIC:	diisopropylcarbodiimide,
	DMF:	dimethylformamide
	HPLC:	high pressure liquid chromatography,
25	MDR1:	product of multidrug resistance gene,
	Me:	methyl,
	Nal:	naphthylalanyl,
	Nle:	norleucyl,
	m.p.:	melting point,
30	OPFP:	pentailuorphenyl
	ON _p :	p-nitrophenyl
	Pen:	penicillinalanyl,
	Phe:	phenylalanyl,
	Phg:	phenylglycyl,
35	Pro:	prolyl,
	R _f :	retention factor,
	SUC:	succinyl,
	TEA:	triethylamine,
	THF:	tetrahydrofuran,
40	Thr:	threonyl,
	Thx:	thyroxyl,
	TLC:	thin layer chromatography,
	Trp:	tryptophyl,
	Z:	benzyloxycarbonyl.

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As preferred embodiments of the invention, X^1 can be BOC, Glu(OBzl)-OBzl, Z, or (D-Phe-Trp-OMe); X^2 can be Asp(OBzl), succinyl, Glu(OBzl), or DBTA; X^3 can be Lys, Glu, Asp, or Phe; X^4 can be Z, OBzl, BOC-Glu(OBzl), BOC-Lys(BOC), or Z-Glu(OtBu); or X^3 and X^4 in combination can be Lys[BOC-Glu(OBzl)]; and X^5 can be OtBu, OBzl, OMe, or Trp-OMe.

Preferred formula (I) compounds of the invention include, but are not limited to, the peptide derivatives

10 BOC-Asp(OBzl)-Lys(Z)-OtBu (Reversin 121); succinyl-bis[Glu(OBzl)-OBzl]; Z-D-Glu(OBzl)-D-Asp(OBzl)-OBzl; DBTA-bis(D-Phe-Trp-OMe); DBTA-[Glu(OBzl)₂]₂; N^α, N^ϵ -bis[BOC-Glu(Bzl)]-Lys-OMe (Reversin 205); BOC-Tyr(Bzl)-Tyr(Bzl)-OMe; BOC-D-Ser(Bzl)-Lys(Z)-OtBu; BOC-Glu(OBzl)-Lys(Z)-OtBu; BOC-Glu(OBzl)-Lys(Z)-OMe; N^α, N^ϵ -bis[BOC-Lys(BOC)]-Lys-OMe; or N^α, N^ϵ -bis[Z-Glu(OtBu)]-Lys-OMe.

Any of the various compounds of the invention can be combined with a pharmaceutically acceptable carrier or an adjuvant. The various compounds of the invention can

20 also be combined with a drug, e.g., a chemotherapeutic, antiparasitic, or antibiotic drug, for convenient co-administration of the Reversin compound and the drug to a patient. The Reversin molecule can, in addition to inhibiting MDR1 activity, also act as an adjuvant to

25 enhance the activity of the drug. As used herein, a "drug", includes a medication, a pharmaceutical, or a substance which is intended for use in diagnosis, cure, mitigation, treatment, or prevention of disease, or which is generally intended to affect the structure or the

30 function of the body of a mammal.

The invention also includes a method of preparing any of the various formula (I) compounds of the invention. The method involves providing a combination of one or more of X^1_n , X^2 , X^3 , X^4_n , X^5 , or X^1X^2 in a

35 solution, e.g., by dissolving the combination, e.g., one

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or more of X^1_n , X^2 , X^3 , X^4_n , X^5 , or X^1X^2 , in a solution; cooling the solution; adjusting the pH of the solution to the neutral range, e.g., a pH of 4-8, preferably pH 6-8, or between pH 7 and 8, inclusive; and purifying the
5 formula (I) compound from the solution. By "purifying" is meant extracting, filtering, evaporating, precipitating, washing, recrystallizing, isolating by chromatography, or any other means of isolating the desired Reversin compound from the reaction mixture. The
10 method can further include an additional purification step to remove any impurities from the final product, e.g., a gel filtration step, or a chromatographic step (see Methods, below). The method can also include, or further include, a step of active ester coupling, or a
15 step of dicyclohexylcarbodiimide condensation characterized by the following parameters: cooling by ice-water, 10% excess of DCC, pH adjusted between 7-8 with tertiary base (e.g. triethylamine, N-methylmorpholine, diisopropyl-ethylamine). In certain
20 cases 1-hydroxybenzotriazole additive is used for activation and to avoid possible racemization (W. Lonig et al. Chem Ber. 103:2024, 1970, hereby incorporated by reference).

The invention also includes a method of reducing
25 the activity of a multidrug transporter protein or its homologues in a mammal. The method involves administering to the mammal an amount of any of the various formula (I) compounds of the invention in a therapeutically effective amount. The method of reducing
30 the activity of MDR1 can be used to lower resistance to a drug, where the drug includes one or more, e.g., at least one, two, or three drugs, which are chemotherapeutic drugs, antiparasitic drugs, or antibiotic drugs. By "mammal" is meant a human, a domesticated animal, e.g., a
35 cat or a dog, or an agricultural animal, e.g., a cow,

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- pig, sheep, horse, or poultry. A "chemotherapeutic drug" includes any drug intended to target and kill a tumor cell, e.g., neoplastic, malignant, or benign tumor cell, in a mammal. An "antiparasitic drug" includes a drug
- 5 intended to target the agent of a parasitic infection, e.g., ascaris, enterobium, hookworm, threadworm, tapeworm, schistosomes, whipworm, protozoa, e.g., intestinal or extraintestinal amebas, giardia, malaria, toxoplasma, or trichomonas. An "antibiotic drug"
- 10 includes substances which inhibit or kill fungal or bacterial microorganisms, e.g., actinomycin. Examples of drugs within the scope of the invention include, but are not limited to, the substances listed in Table 1, as well as any chemotherapeutic, antiparasitic, and antibiotic
- 15 drugs which clinically elicit, or whose therapeutic effects are limited by, primary or secondary multidrug resistance caused by MDRI (Ford et al. *supra*; hereby incorporated by reference).

- Cytotoxic drugs which are extruded by the MDRI
- 20 protein or its homologues include, but are not limited to, the compounds shown in Table 1.

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Table I.

Drugs exported by the MDR1 protein

<u>Anti-Cancer Drugs</u>	<u>Examples</u>
5 Vinca alkaloids	vinblastine, vincristine vindesine
Anthracyclines	doxorubicin, daunorubicin epirubicin
Epipodophyllotoxins	etoposide
10 Antibiotics	actinomycin D
Others	mitomycin C, taxol, topotecan, mithramycin
<u>Other cytotoxic agents</u>	<u>Examples</u>
15 Anti-microtubule drugs	colchicine, podophyllotoxin
Protein Synthesis inhibitors	puromycin, emetine
DNA intercalators	ethidium bromide
Toxic peptides	valinomycin, gramicidin D, N-acetyl-leucyl- leucyl-norleucinal (ALLN)
20	

The method of reducing the activity of a MDR1 protein or its homologues in a mammal is also used to facilitate administration of a drug through membranes which exclude various substances from a given type of cell or tissue. In particular, Reversins can be used to aid transport of a drug through the blood-brain barrier, or through the blood-testis barrier. By "blood-brain barrier" and "blood-testis barrier" is meant the endothelial lining of cells that are selectively permeable or impermeable to substances circulating outside of the brain or testis, respectively.

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A "multidrug transporter protein" (MDR1), as used herein, refers to a glycoprotein present on the membrane of many cell types which acts to extrude various substances from the cell. In humans, MDR1 is commonly referred to as the P-glycoprotein, P-170, or Pgp, and is encoded by the mdr1 gene. Also included are homologues of MDR1 which are members of the MDR1 family of proteins in other organisms, e.g., prokaryotes or lower eukaryotes, e.g., bacterial, yeast, fungal, parasitic organisms, or other organisms that take up residence within a mammalian body. Homologues within the MDR1 family of proteins perform the same function as MDR1 for cells of the other organisms, i.e., by transporting hydrophobic cytotoxic compounds out of the cell. Generally, the method of the invention is intended to inhibit the activity of members of the MDR1 protein family which are shown by the assays below to be affected by a Reversin compound.

A "therapeutically effective amount", as used herein, refers to an amount that is effective at reducing the activity of the multidrug transporter protein; an amount that is effective at lowering resistance of the mammal to a drug or to a group of drugs; or an amount that is effective for facilitating absorption of a drug through the blood brain barrier. An "effective amount" can be calibrated by the assays below. By "facilitating" is meant enhancing the overall amount of the drug that is absorbed, or the fraction of the drug that is absorbed.

As used herein, the term "reducing" means either partially or completely inhibiting MDR1 activity. By "reducing" is also meant decreasing, lowering, or overcoming the effects of drug or multidrug resistance, so that less drug is transported from the target cell, or so that a greater concentration of drug accumulates within the cell. As used herein, the term "reducing"

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encompasses both treating and preventing the occurrence of drug, e.g., multidrug, resistance in a mammal. The level of MDR1 activity present in a cell or in a target tissue is measured by the assays provided below. This in turn permits accumulation of drug at higher concentrations in the cell than would be possible in the absence of the Reversin compound.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Detailed Description

We first briefly describe the drawings.

Drawings

Fig. 1 is a schematic illustration of the MDR1 protein in its membrane environment.

Fig. 2 is a computer-generated illustration of the secondary structure of the Reversin 121 molecule, BOC-Asp(OBzl)-Lys(Z)-OtBu.

Fig. 3 is a graph showing drug-stimulation of human MDR1-ATPase activity in isolated insect cell membranes.

Fig. 4 is a graph showing stimulation of the vanadate-sensitive human MDR1-ATPase activity in the isolated membranes of Sf9 cells by verapamil and Reversins.

Fig. 5 is a bar graph showing the effects of Reversins on Fura-2 uptake in NIH 3T3 fibroblasts.

Fig. 6 is a bar graph showing the inhibition of Fura-2 AM loading in MDR1-expressing fibroblasts by agents interacting with MDR1.

Fig. 7 is a photograph showing the effect of verapamil on dye loading in MDR1-expressing fibroblasts using single-cell imaging for fluorescent dye uptake. The upper panel shows control NIH 3T3 cells; middle and

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lower panels show MDR1-3T3 cells. In the lower panel the medium also contained 25 μ M verapamil.

Fig. 8 is a graphic illustration of the fluorescent dye uptake of control cells (A) versus 5 fluorescent dye uptake in the presence of 5 μ M Reversin 205 (B).

Fig. 9 is a bar graph showing the effect of various pretreatments and washings on fluorescent dye uptake into 3T3-MDR cells.

10 Figs. 10A and 10B are graphic illustrations of the effect of Reversin 205 and verapamil on drug-sensitive and multidrug resistant cultured human tumor cells (K562) treated with adriamycin (A) or vincristine (B). \square -K562 control, $+-$ K562 + 5 μ MR, \times -K562 + 10 μ M Verap, \blacksquare -K562
15 MDR, \rightarrow -MDR + 5 μ MR, \blacksquare -K562 MDR + 10 μ MV.

Figs. 11A and 11B are graphic illustrations of the effect of Reversins 121 (A) and 205 (B) on the vinblastine-sensitivity of drug-sensitive (KB3) and multidrug-resistant (KBV1) cultured human tumor cells. \square -
20 KB3 control, $+-$ KB3 + 2 ug/ml R, \times -KB3 + 10 ug/ml R, \blacksquare -KBV1 control, \rightarrow -KBV1 + 2 ug/ml R, \blacksquare -KBV1 + 10 ug/ml R.

Reversin Compounds

The aim of the present invention is to provide chemical reversing agents, or chemosensitizers, to
25 address the clinical problem of multidrug resistance. Toward this end applicants designed a set of novel hydrophobic peptide-based molecules called Reversins.

Reversins effectively inhibit expulsion of therapeutic drugs. They are effective at low
30 concentration, non-toxic, and reversible. Reversins also leave the organism without adverse side effects, and in some cases even enhance the activity of the drug therapy itself. They can be prepared in high purity, in large quantities, and at relatively low cost. Reversins are
35 especially advantageous for preventing resistance against

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anticancer drugs, without causing serious side-effects in other tissues, and without irreversibly disrupting the important natural physiological function of the MDR1 transport protein itself.

5 Methods of Preparation

Reversins are peptide derivatives consisting of naturally occurring L amino acids with bulky aromatic or alkyl groups, and carboxamide or carboxylic acid ester groups. The hydrophobic side-chains of the molecules
10 enhance their interaction with the MDR1 transporter for which they are a substrate. The overall size of the molecule also influences this interaction. Dipeptides and tripeptides are preferred. A computer-analyzed secondary structure of a preferred dipeptide, Reversin
15 121, is illustrated in Fig. 2.

In general, Reversins are synthesized by an appropriate fragment condensation reaction, depending on the chemical character of the amino acid moiety in the compound being prepared. Reversins are synthesized by
20 traditional stepwise condensation methods (The Peptides: Methods of peptide synthesis, Eds. E. Schoder, K. Lubke, Acad. Press, NY, 1966; The Peptides: Analysis, synthesis and biology, Ed. by E. Gross, J. Meienhofer, Acad. Press, NY 1979, each hereby incorporated by reference), or by
25 automated solid-phase peptide synthetic methods, which are relatively large-scale and inexpensive. Reversins are also prepared by methods known to those of ordinary skill in the art, and as exemplified by Examples 1-12, below.

30 Each of the Examples 1-12, below, are prepared from amino acids that were protected to a desired grade. The starting materials are dissolved in apolar solvents, e.g., DMF, acetonitril, or DMSO. Condensation reactions are performed in solution, or by passage of the solution
35 over a solid-phase column. The solution is cooled, for

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example to 0-10°C, and the pH is adjusted to the neutral range with, e.g., triethylamine, N-methylmorpholine, trimethylamine, or diisopropylethylamine, and the product is allowed to form slowly, e.g. by stirring overnight.

- 5 Finally, the Reversin compound is purified from the reaction mixture by, e.g., filtration of the precipitate, or by evaporating away the mother liquor, and the product is washed.

- The final products can receive additional
- 10 purification in order to remove any contaminants from the Reversin compound. The final purification step can include one or a combination of the following procedures: ethyl acetate; gel filtration; preparative high pressure liquid chromatography (HPLC); medium pressure column
- 15 chromatography; or silica gel column chromatography. The purity of the peptides obtained is determined by thin layer chromatography (TLC) analysis.

- In the examples that follow, R_f values were obtained by TLC on Kiesel gel sheets (DC Alufolien Merck)
- 20 using the following solvent mixtures (in the examples the solvent mixtures will be identified by the numbers listed below):

- (1) acetone:toluene, 1:1
- (2) chloroform:acetic acid:benzene, 85:10:5
- 25 (3) acetic acid:benzene, 1:7
- (4) ethyl acetate:pyridine:acetic acid:water
240:20:6:11.

- In general, Reversins are only slightly soluble in water (maximum solubility is about 10 µg/ml), while
- 30 freely soluble in dimethyl sulfoxide (DMSO), glycerol, or ethanol. When testing a Reversin in an *in vitro* cellular screening assay, the presence of serum in the culture media increases the solubility of the Reversin.

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The following non-limiting examples are provided to illustrate methods of preparing the Reversin compounds of the invention.

Example 1

5 **Preparation of BOC-Asp(OBzl)-Lys(Z)-OtBu (Reversin 121)**

After dissolving 1 mmol of BOC-Asp(OBzl)-OH amino acid derivative (mole wt. = 323) in 50 ml of DMF, 1.1 mmoles of DCC (mole wt. = 206) and then 1 mmole of N^ε-carbobenzoxyllysine tert.-butyl ester hydrochloride (mole
10 wt. = 371) were added while stirring and cooling with ice water. The pH value of the reaction mixture was adjusted to 7-8 with triethylamine and the mixture was stirred at room temperature overnight. The precipitate was filtered off and the mother liquor was evaporated. After taking
15 up the residue in 150 ml of ethyl acetate, the extract was washed three times with 120 ml of 10% citric acid solution each, then three times with saturated sodium hydrogen carbonate solution, and finally three times with saturated saline solution. After drying the ethyl
20 acetate phase over anhydrous sodium sulfate and filtering off the drying agent, the organic phase was evaporated and the residue was recrystallized from 30% alcohol to give the title product. m.p.: 128-129°C, R_f (4) = 0.9. The possible conformation of Reversin 121 is shown in
25 Fig. 2.

The molecular mass of Reversin 121 is 641.5.

Example 2

Preparation of succinyl-bis[Glu(OBzl)-OBzl]

To a solution containing 1 mmole of dibenzyl
30 glutamate hydrochloride (mole wt. = 362) in 50 ml of DMF, 0.5 mmole of bis(pentafluorophenyl) succinate was added while stirring and cooling with ice water. The pH value of the reaction mixture was adjusted to between 7 and 8 with triethylamine, and the mixture was stirred at room
35 temperature overnight. Then the precipitate was filtered

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off, the mother liquor was evaporated and the residue was taken up in 150 ml of ethyl acetate. The organic solution was successively washed three times with 120 ml of 10% citric acid solution each, three times with
5 saturated sodium hydrogen carbonate solution, and finally with saturated saline solution. After drying the ethyl acetate the phase over anhydrous sodium sulfate and filtering off the drying agent, the organic phase was evaporated, and the evaporation residue was recrystallized
10 from aqueous alcohol to give the white crystalline title product, m.p.: 106-107°C, R_f (4) = 0.9.

Example 3

Preparation of Z-D-Glu(OBzl)-D-Asp(OBzl)-OBzl

To a solution containing 1 mmol of the amino acid
15 derivative Z-D-Glu(OBzl)-OH (mole wt. - 371) in 50 ml of DMF, 1.1 mmoles of DCC and then 1 mmol of H-D-Asp(OBzl)-OBzl hydrochloride (mole wt. - 349) were added while stirring and cooling with ice water. The pH value of the reaction mixture was adjusted to between 7 and 8 with
20 triethylamine, and the mixture was stirred at room temperature overnight. After filtering off the precipitate, the mother liquor was evaporated, and the residue was taken up in 150 ml of ethyl acetate. The organic solution was successively washed three times with
25 120 ml of 10% citric acid solution each, three times with saturated sodium hydrogen carbonate solution, and finally with saturated saline solution. After drying the ethyl acetate phase over anhydrous sodium sulfate, the drying agent was filtered off and the organic phase was
30 evaporated to give the title compound as a slightly yellow crystalline product. R_f (4) = 0.9.

Example 4

Preparation of DBTA-bis(D-Phe-Trp-OMe)

To a solution containing 1 mmole of the dipeptide
35 hydrochloride D-Phe-Trp-OMe (mole wt. = 401.8) in 50 ml

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of DMF, first 1.1 mmoles of DCC, and then subsequently 0.5 mmole of DBTA-(OPFP)₂ active ester (mole wt. = 696), were added while stirring and cooling with ice-water. The pH value of the reaction mixture was adjusted to
5 between 7 and 8 with triethylamine and the mixture was stirred at room temperature overnight. Then, the precipitate was filtered off, the mother liquor was evaporated, and the residue was taken up in 150 ml of ethyl acetate. The organic solution was successively
10 washed three times with 120 ml of 10% citric acid solution each, 3 times with saturated sodium hydrogen carbonate solution, and finally three times with saturated saline solution. After drying the ethyl acetate phase over anhydrous sodium sulfate and filtering
15 off the drying agent, the organic phase was evaporated to obtain the title compound as a foam-like amorphous evaporation residue. m.p.: 79-80°C, R_f (4) = 0.9.

Example 5

Preparation of DBTA-[Glu(OBzl)₂]₂

20 To a solution containing 1 mmole of the active ester DBTA-(OPFP)₂ (mole wt. = 696) in 50 ml of DMF, 2 mmoles of Glu(OBzl)₂ tosylate (mole wt. = 500) and then 0.28 ml (2 mmoles) triethylamine, were added while stirring and cooling with ice water. The pH value of the
25 reaction mixture was adjusted to between 7 and 8 with triethylamine and the mixture was stirred at room temperature overnight. Then, the precipitate was filtered off and after evaporating the mother liquor the residue was taken up in 150 ml of ethyl acetate. The
30 organic solution was successively washed three times with 120 ml of 10% citric acid solution each, three times with saturated sodium hydrogen carbonate solution, and finally three times with saturated saline solution. After drying the ethyl acetate phase over anhydrous sodium sulfate,
35 the drying agent was filtered off and the organic phase

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was evaporated to give the title compound as an oily residue. R_f (4) = 0.9.

Example 6

Preparation of N^α , N^ϵ -bis[BOC-Glu(OBzl)]-Lys-OMe (Reversin

5 205)

Method A: To a solution containing 1 mmol of the amino acid derivative BOC-Glu(OBzl)-OH (mole wt. = 337) in 50 ml of DMF, 1.1 mmoles of DCC and 1 mmoles of the amino acid derivative Lys-OMe dihydrochloride (mole wt. = 10 228) were added while stirring and cooling in ice water. After adjusting the pH value to between 7 and 8 with triethylamine, the reaction mixture was stirred at room temperature overnight. Then, the precipitate was filtered off and the mother liquor was evaporated. The 15 residue was taken up in 150 ml of ethyl acetate and successively washed three times with 120 ml of 10% citric acid each, three times with saturated sodium hydrogen carbonate solution, and finally three times with saturated saline solution. After drying the ethyl 20 acetate phase over anhydrous sodium sulfate, the drying agent was filtered off and the organic phase was evaporated. After recrystallizing the evaporation residue from the aqueous alcohol, the title compound was obtained as a slightly yellow crystalline product. m.p.: 25 79-81°C, R_f (1) = 0.7, R_f (4) = 0.95.

Method B: A second method of preparing N^α , N^ϵ -bis[BOC-Glu(OBzl)]-Lys-OMe is according to the procedure of Example 11, except that 5.03 g of the amino acid active ester BOC-Glu(OBzl)-OPFP was used as the starting 30 material, and the other constituents were used in the amounts of 1.15 g of Lys-OMe dihydrochloride, 20 ml of DMF and 1.38 ml of triethylamine.

The molecular mass of Reversin 205 is 875.5.

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Example 7**Preparation of BOC-Tyr(Bzl)-Tyr(Bzl)-OMe**

To a solution containing 986 mg of the amino acid derivative BOC-Tyr(Bzl)-ONp (mole wt. = 491) in 50 ml DMF, first 682 mg of H-Tyr(Bzl)-OMe. OMe hydrochloride salt (mole wt = 384), and then 0.28 ml triethylamine were added while stirring and cooling with ice water. The pH value of the reaction mixture was adjusted to between 7 and 8 with triethylamine, and the mixture was stirred at room temperature for 24 hours. After evaporating the mother liquor the residue was taken up in 50 ml of ethyl acetate. The organic solution was successively washed three times with 2N KHSO₄ solution, three times with saturated sodium hydrogen carbonate solution, and three times with saturated saline solution. After drying the ethyl acetate phase over anhydrous sodium sulfate, the drying agent was filtered off. The organic phase was evaporated to give an oil which can be crystallized from the mixture of ethanol:water (7:3) to obtain the title compound. $R_f(1):0.95$; $(R_f(4):0.9$; m.p.=161-163 °C.

Example 8**Preparation of BOC-D-Ser(Bzl)-Lys(Z)-OtBu**

To a solution containing 10 mmoles of the amino acid derivative BOC-D-Ser(Bzl)-OH (mole wt. = 294) in 20 ml DMF, were first added 11 mmoles of DCC (mole wt. = 206), 3.5 g of H-Lys(Z)-OtBu hydrochloride salt (mole wt. = 336). Then 1.13 ml of triethylamine were added while stirring and cooling in ice water. The pH value of the reaction mixture was adjusted to between 7 and 8 by using triethylamine, and the mixture was stirred at room temperature for 24 hours. After evaporating the mother liquor the residue was taken up in 50 ml of ethyl acetate. The organic solution was successively washed three times with 2N KHSO₄ solution, three times with saturated sodium hydrogen carbonate solution, and finally

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three times with saturated saline solution. After drying the ethyl acetate phase over anhydrous sodium sulfate, and filtering off the drying agent the organic phase was evaporated to obtain the title compound as an oil which
5 is triturated in petroleum ether. $R_f(4):0.85$; $R_f(5):0.65$.

Example 9

Preparation of BOC-Glu(OBzl)-Lys(Z)-OtBu

To a solution containing 2.12 g of BOC-Glu(OBzl)-OFFP amino acid active ester (mole wt. = 503) in 20 ml
10 DMF, first 744 mg H-Lys(Z)-OtBu hydrochloride salt (mole wt. = 371), then 0.28 ml triethylamine were added while stirring and cooling by ice-water. The pH value of the reaction mixture is adjusted between 7 and 8 by using triethylamine and the mixture is stirred at room
15 temperature for 24 hours. After evaporating the mother liquor the residue is taken up in 30 ml of ethyl acetate. The organic solution is successively washed three times with 10% citric acid, three times with saturated sodium hydrogen carbonate solution, and finally three times with
20 saturated saline solution. After drying the organic phase over anhydrous sodium sulfate, the drying agent was filtered off and the organic phase was evaporated to give an oil which was then triturated in petroleum ether and crystallized from ethanol with water to obtain the title
25 compound. $R_f(1):0.95$; m.p.=79-81 °C.

Example 10

Preparation of BOC-Glu(OBzl)-Lys(Z)-OMe

To a solution containing 2.1 g of the amino acid active ester BOC-Glu(OBzl)-OPFP (mole wt. = 503) in 10 ml
30 DMF, was first added 1.3 g of H-Lys(Z)-OMe hydrochloride salt (mole wt. = 329). Then 0.28 ml of triethylamine were added while stirring and cooling with ice water. The pH value of the reaction mixture was adjusted to between 7 and 8 with triethylamine, and the mixture was stirred at
35 room temperature for 24 hours. After evaporating the

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mother liquor the residue was taken up in 50 ml of ether. The organic solution was successively washed three times with 2N KHSO₄ solution, three times with saturated sodium hydrogen carbonate solution, and finally three times with
5 saturated saline solution. After drying the organic phase over anhydrous sodium sulfate, the drying agent was filtered off, and the organic phase was evaporated to give an oil which was triturated in petroleum ether and crystallized from ethanol with water to obtain the title
10 compound. R_f(1)=0.80; R_f(4):0.85; R_f(5):0.90; m.p. = 102-105°C.

Example 11

Preparation of N^α,N^ε-bis[BOC-Lys(BOC)]-Lys-OMe

To a solution containing 5.12 g of the amino acid
15 active ester BOC-Lys(BOC)-OPFP (mole wt. = 512) in 50 ml DMF, was first added 1.15 g of Lys-OMe dihydrochloride salt (mole wt. = 231). Then 1.38 ml of triethylamine were added while stirring and cooling with ice water. The pH value of the reaction mixture was adjusted to between 7
20 and 8 with triethylamine, and the mixture was stirred at room temperature for 24 hours. After evaporating the mother liquor the residue was taken up in 50 ml of ether. The organic solution was successively washed three times with 2N KHSO₄ solution, three times with saturated sodium
25 hydrogen carbonate solution, and finally three times with saturated saline solution. After drying the organic phase over anhydrous sodium sulfate, the drying agent was filtered off, and the organic phase was evaporated to give the title compound as an oil. R_f(1):0.90; R_f(4):0.90.

30

Example 12

Preparation of N^α,N^ε-bis[Z-Glu(OtBu)]-Lys-OMe

N^α,N^ε-bis[Z-Glu(OtBu)]-Lys-OMe was prepared according to the procedure of example 11, except that 2.5 g of the amino acid active ester Z-Glu(OtBu)-OPFP was

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used as the starting material, and other constituents were used in the amounts of 0.6 g of Lys-OMe dihydrochloride, 25 ml DMF, and 0.1 ml triethylamine. The title compound is a white crystalline material.

5 $R_f(1):0.70$; $R_f(4):0.75$; m.p. = 83-84 °C.

Methods for Demonstrating the Efficacy of Reversins

Any of the various Reversin compounds of the invention can be screened for their ability to reduce the activity of the MDR1 protein according to the following
10 *in vitro* and *in vivo* methods. In addition to the instructions and experiments provided below, each method is supported by one or more publications, each of which is hereby incorporated by reference.

A. *In vitro* Methods

15 Two test systems were developed to specifically assess the ability of a Reversin compound to interact with the human MDR1 protein. The first system measures the ATPase activity of MDR1, while the second system measures the level of a fluorescent indicator extruded by
20 the MDR1 protein.

1. ATPase Assay

In the first assay, MDR1-ATPase activation reflects the interaction and relative affinity MDR1 has for a candidate compound. The MDR1-ATPase is stimulated
25 by cytotoxic drugs, e.g., vincristine, while it is insensitive to chemicals that are not transported by the MDR1 protein. The MDR1-ATPase is also stimulated by known chemosensitizing agents, such as the multidrug-resistance reversing agents verapamil and quinine (Fig.
30 3), probably competing with drug extrusion of the multidrug transporter. Thus, by measuring this MDR1-ATPase a relatively simple *in vitro* assay system became available for assessing direct drug interactions with the

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MDR1 protein (Sarkadi et al. *J. Biol. Chem.* 267:4854-4858, 1992).

The assay was developed by expressing the human MDR1 protein in *Spodoptera frugiperda* insect cells. The 5 cultured cells were infected with a baculovirus into which the cDNA of human MDR1 was genetically engineered. The recombinant virus-infected cells produce a large amount of the MDR1 protein, properly folded and functionally inserted into the membrane. Additional 10 details regarding construction of these recombinant strains are provided by Germann et al. (*Biochemistry* 29:2295-2303, 1988) and Sarkadi et al. (1992 *supra*).

Measurements of the effect of Reversins on the MDR1 ATPase activity are performed as follows, and 15 according to the methods provided by Sarkadi et al. (1992, *supra*).

MDR1 ATPase measurements:

Spodoptera frugiperda (Sf9) cells were infected with a recombinant baculovirus carrying the human MDR1 20 gene, and cultured according to the procedures described previously (Germann et al. 1990 *supra*; Sarkadi et al. 1992 *supra*). The virus-infected Sf9 cells were harvested, and their membranes isolated and stored as described (Sarkadi et al. *J. Biol. Chem.* 267:4854-4858, 25 1992). The amount of ATP consumption measured in these membranes reflects the ATP-dependent transport function of the multidrug transporter. ATPase activity of the isolated Sf9 cell membranes was estimated by measuring inorganic phosphate (P_i) liberation. To do this, a 30 membrane suspension (about 10 μ g of membrane protein) was incubated at 37°C in 0.1 ml of a medium containing 50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM DTT, 50 mM KCl, and 5 mM Na-azide. The ATPase reaction was started by the addition of 5 mM MgATP. The reaction was stopped by the 35 addition of 0.1 ml of 5% SDS solution, and the amount of

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P_i determined immediately. ATPase activity was estimated by the difference obtained in P_i levels by a sensitive colorimetric assay between zero minutes (reaction stopped immediately with SDS) and 20 minute incubation periods.

- 5 The data points show the means of triplicate determinations in a representative experiment. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μ M) are plotted. Isolated membranes of uninfected or B galactosidase
10 infected Sf9 cells had no drug-stimulated ATPase activity (Fig. 3).

When Reversins 121 and 205 were tested in the ATPase assay system, they greatly stimulated the MDR1-ATPase, but were effective at significantly (one to two
15 orders of magnitude) smaller concentrations than the known reversing agents verapamil and quinine. The half-maximal activating concentration (K_a) of verapamil was approximately 1 μ M, while the K_a value for Reversin 121 was approximately 60 nM and for Reversin 205 this value
20 was about 30 nM (Fig. 4). Thus, the multidrug transporter seems to interact with Reversins with an exceptionally high affinity. As shown in Fig. 4, a strong inhibition of the MDR1-ATPase was observed at higher concentrations (above 1 μ M) of Reversin 205.

25 2. Fluorescence Assay:

The second assay is based on the measurement of fluorescent dye uptake into intact cells. Fluorescent dyes are often used to indicate intracellular calcium or pH changes. An effective technique for cellular dye
30 loading is the application of acetoxymethyl ester (AM) derivatives. These hydrophobic dye esters are non-fluorescent outside the cell are cleaved by intracellular esterases into hydrophilic fluorescent free acids. This intracellular "trapping" of the free dye and the
35 continuous inward gradient of the AM compounds results in

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the accumulation of large amounts of fluorescent indicator inside the cell.

Cell culturing:

NIH 3T3 cells were cultured under standard conditions in D-MEM medium. MDR1-transfected cells (NIH MDR1 G185) were prepared and characterized for their drug-resistant properties as described (Ambdukar et al. *Proc. Natl. Acad. Sci. USA* 89:8472-8476, 1992; Bruggemann et al. *J. Biol. Chem.* 267:21020-21026, 1992; Sarkadi et al. 1992 *supra*). Before each experiment the cells were trypsinized, then washed and stored in D-MEM medium at 37°C. KBV1 (MDR1⁺) and KB3 (MDR1⁻) human tumor cells were also cultured in D-MEM, which K562 human tumor cells were grown in RPMI medium, supplemented with 10% FCS.

The effects of Reversins 121 and 205 on MDR1 function in intact cells was examined by a fluorescent dye extrusion assay (Fig. 5). Mouse NIH 3T3 fibroblasts, transfected with human MDR1 cDNA and expressing human MDR1 protein, actively extrude the hydrophobic AM derivatives of fluorescent dyes, e.g., Fura2-AM (Homolya et al. *J. Biol. Chem.* 29:21493-21496, 1993; Hollo et al. *Bioch. Biophys. Acta.* 1191:384-388, 1994). Similar experiments can be performed with MDR1-expressing human tumor cells. Verapamil, vincristine, and Reversins 121 and 205 inhibit this dye extrusion, most probably by competing with the dye on the transporter. In the experiments shown here maximally effective concentrations of Reversins were used. However, Reversins also act in at least one order of magnitude smaller concentrations than verapamil.

Human MDR1-transfected mouse fibroblasts and MDR1-expressing human tumor cells actively extrude the AM forms of several fluorescent indicators, lowering the level of intracellular fluorescence in cells with active multidrug transport. Thus the accumulation in such cells

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of fluorescent dye is strongly inhibited. This MDR1-specific dye-AM extrusion is blocked by competing substrates and inhibitors of the MDR1 transporter, e.g., by verapamil, vincristine, sodium orthovanadate, and a
5 monoclonal anti-MDR1 antibody. In contrast, these agents have no effect on dye accumulation in fibroblasts which do not overexpress MDR1 (Fig. 6). See also Kessel et al. (Cancer Res. 51:665-670, 1991); Neyfakh et al. (Exp. Cell. Res. 174:168-176, 1988); and Sarkadi et al. (J.
10 Biol. Chem. 268:21493-21496, 1993).

Fluorescence studies:

Dye uptake was measured by incubating 2×10^6 cells/ml D-MEM medium at 37°C in the presence of 0.5 μ M Fura-2 AM (added in 5 mM stock solution in DMSO), then
15 rapidly spinning the cells (15 sec, 12,000 x g), and rinsing the pellet with HPM1 medium (containing 120 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 0.04 mM CaCl₂, 10mM HEPES-Na (pH 7.4), 10 mM NaHCO₃, 10 mM glucose, and 5 mM Na₂HPO₄). The cells were resuspended in 2 ml HPM1 and fluorescence
20 was measured with rapid stirring in a Hitachi F-4000 fluorescent spectrophotometer. The excitation wavelength was 340 nm, and emission was measured at 410 nm. Maximum fluorescence and dye concentration were measured after the addition of 0.5% Triton X-100 and 2 mM CaCl₂ to the
25 medium. The dye concentration was calibrated based on the measurements of free acid dye fluorescence in the same instrument under identical conditions.

Flow-cytometric measurements:

Fluorescence measurements were done in 60 sec
30 scanning periods using a Cytoronabsolut instrument (Ortho Diagnostic Systems, NJ). The excitation was set to 488 nm. Green fluorescence was measured with a filter with a range of 515-548 nm, while red fluorescence was measured above 620 nm.

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With this method drug-interactions with the MDR1 protein can be measured by following cellular fluorescence allowing a flow cytometric or single cell imaging detection of the function of MDR1 in tumor cells.

5 As shown in the single cell images of Fig. 7, fibroblasts expressing the MDR1 protein, in contrast to the control cells, are not loaded with a fluorescent dye, while verapamil, which inhibits the multidrug transporter, restores dye uptake.

10 When assaying the effect of Reversin molecules on the fluorescent dye uptake and the drug-resistance in various MDR1-transfected and multidrug-resistant tumor cell lines *in vitro*, these experiments indicated a strong inhibitory action on drug extrusion by low concentrations
15 of Reversins, again showing a specific interaction of the multidrug transporter with these molecules.

Binding Experiments:

The relative ability of Reversins to bind to the MDR1 protein, or to be removed from the cell membrane, is
20 measured by "wash-out experiments" (Fig. 9). NIH 3T3-MDR1 cells were pre-treated with 15 μ M verapamil, 5 μ M Reversin 121, or 5 μ M Reversin 205 for 5 minutes, which produced a complete inhibition of dye extrusion by MDR1 with 1% serum in the media. The cells were washed once
25 with the standard incubation media and a 5 min centrifugation at 800 x g. Dye uptake was then measured with or without the addition of 15 μ M verapamil during the uptake period. As shown, preincubation with verapamil had no effect on dye uptake, while both
30 Reversin 121 and Reversin 205 had a major effect even after washing the cells. Thus verapamil could be eliminated by a single wash, while 121 and 205 remained effectively bound to MDR1.

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*Assessing Reversin activity by its ability
to enhance the cytotoxicity caused by
other agents*

Another method for assessing the ability of
5 Reversins to inhibit drug resistance is to measure the
cytotoxicity of drugs in multidrug-resistant human tumor
cell lines. Since MDR1 normally lowers the concentration
of cytotoxic agents to subtoxic concentrations by
extruding them from the cell, inhibition of MDR1 would be
10 expected to enhance cytotoxicity.

In these studies, adriamycin, vincristine, and
vinblastine, in originally ineffective concentrations,
become effectively cytotoxic in the presence of 1-10
 $\mu\text{g/ml}$ of Reversins 121 or 205. Figs. 10A and 10B present
15 such an experiment for Reversin 205 using K562 human
erythroblastoid tumor cells and their adriamycin-selected
multidrug-resistant subline. Figs. 11A and 11B are
similar experiments with the intestinal tumor cells KB3
and KBV1, the latter being a multidrug resistant subline.
20 In the experiment shown in Fig. 11(A), Reversin 121 was
found to be cytotoxic in the MDR1-expressing cells (but
not in the control cells) even without the addition
vinblastine. Such a collateral toxic effect (which may
be due to the ATP-consuming futile functioning of the
25 drug transporter) can be greatly advantageous in treating
drug-resistant tumors.

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Application of the in vitro fluorescent assay for clinical diagnosis

By using the some fluorescent dye extrusion assay described above the in vitro effects of Reversins on the
5 multidrug-resistant leukocytes of a leukemic patient were studied. Cells were isolated by withdrawing blood from the patient, and isolating white blood cells by centrifugation. Cells can be isolated from other types of tumors by biopsy. A flow cytometer was used to
10 measure fluorescent dye loading of individual cells, which had been previously shown to express MDR1, in the absence or presence of Reversin 205 (Fig. 8). The uptake of fluorescent dye under these conditions models the uptake of cytotoxic drugs into the tumor cells.
15 Fluorescent dye uptake was measured at 37 °C for 10 minutes. In Fig. 8, the MDR1 containing cells in the control experiment had a low fluorescent dye uptake (A), while the addition of 5 μ M Reversin 205 blocked dye extrusion by MDR1 (B). Reversin 205 thereby
20 significantly increased dye uptake and yielded a uniformly high fluorescence in the leukocytes. Further details for the method are provided by Hollo et al., *supra*.

Addition in vitro methods:

25 Additional in vitro methods of screening the ability of a Reversin compound to act as a chemosensitizing agent for the reversal of multidrug resistance are provided by Ford et al. *supra*, at Tables 1-6.

30 B. In vivo Methods

In vivo animal studies for the effectiveness of Reversins can be conducted using any suitable animal model system known to those skilled in the art. One example of an appropriate system is the mouse P388
35 leukemia model system (Tsurouo et al., *supra*). This

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animal model is widely accepted for testing the effect of cytotoxic antileukemic agents or response-modifier compounds.

Inbred (DBA x black F1) mice received 10^6 P388 leukemia cells by intraperitoneal injection. The survival of the mice was followed. The mice were injected with control P388 cells, as well as with P388 cells that had been selected under drug exposure. Drug exposure induced over-expression of the MDR1 protein (P388-MDR cells).

In one trial, the mice had a mean survival time of 14-16 days (they die in a generalized leukemia caused by the P388 cells). The mice injected with the P388 cells were treated with doxorubicin (adriamycin) in a dose of 1 mg/kg/day for 6 days after the injection of the P388 cells. The results showed that adriamycin prolonged the survival time in the case of mice injected with control P388 cells, exceeding the period of 30 days. In contrast, adriamycin therapy did not significantly prolong the lifetime of mice injected with P388-MDR cells. Reversin can be administered to the animals in a dose of 2 mg/kg, a level which has no toxic effect on the control animals, as discussed above.

Another test system is to conduct similar trials using human xenografts in nude mice. Human erythroleukemia (K562) cells are injected into tolerant mice. The developing leukemia is treated with cytotoxic compounds with or without a Reversin candidate compound.

Another animal model system for testing the ability of a Reversin compound to reverse multidrug resistance is to use a transgenic mouse which expresses the human MDR1 gene, e.g., in its bone marrow (Pastan et al. FASEB Jour. 5:2523-2528, 1991).

Human clinical trials can be performed according to methods known to those skilled in the art. For

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example, Dalton et al. provide methods of testing the chemosensitizer verapamil for its ability to modify resistance to a chemotherapeutic (*Jour. Clin. Oncol.* 7:415-424, 1989). Additional methods for conducting

5 human clinical trials of Reversins include, but are not limited to, those provided by Berenbaum et al. (*Pharmacol. Rev.* 41:93-141, 1989); Benson et al. (*Cancer Treat. Rep.* 69:795-799, 1985); Cairo et al. (*Cancer Res.* 49:1063-1066, 1989); Fine et al. (*J. Clin. Oncol.* 5:489-

10 495, 1987); Frishman et al. (*J. Cardiol.* 50:1180-1184, 1982); Miller et al. (*J. Clin. Oncol.* 6:880-888, 1988); Ozols et al. (*J. Clin. Oncol.* 5:641-647, 1987); and Presant et al. (*Am. J. Clin. Oncol.* 9:355-357, 1986).

Therapeutic Use of Reversins

15 The Reversins of the invention can be used therapeutically to inhibit the *in vivo* activity of the MDR1 protein in a patient experiencing drug resistance or poor drug absorption. An effective amount of the Reversin can be administered intravenously to the

20 patient, or administered orally according to conventional methods, in the form of a capsule, liquid, tablet, powder, or pill. Reversin can also be incorporated into an implanted or orally administered slow release device.

 Reversin can be prepared for therapeutic use by

25 mixing the compound with pharmaceutical carriers and/or additives that aid solubility, absorption, flavor, or texture to the vehicle or its contents, e.g., physiological saline, oil, e.g., refined soy bean oil, gelatin, glycerin, or purified water.

30 An appropriate dosage is between 50 $\mu\text{g/kg}$ and 100 mg/kg. An effective and safe dosage can be determined by conventional methods or by the methods taught herein, or by calibration to a given patient on an individual basis.

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It has been found that compounds of the formula (I) according to our invention are capable of stimulating, in a concentration of 0.03 μ M, or of inhibiting, respectively, in concentrations of 1 to 5 μ M, the activity of the MDR1 protein. The stimulating concentrations are by 1 to 3 orders, the inhibitory concentrations are by 1 to 2 orders lower than the corresponding concentrations of substances known from the literature, e.g., verapamil.

10 The safety of Reversins for human administration can be confirmed in appropriate animal models. For example, the *in vivo* acute and subacute toxicity of Reversins was examined in laboratory rats. Reversins 121 and 205 solutions were prepared and administered as follows. Type A solutions contained 50 μ g/ml Reversin 121 or 205 in 20% ethanol in physiological saline. Type B solutions contained 1 mg/ml in glycerol, containing 10% ethanol. Type A solutions (0.5 ml) were administered intravenously to three laboratory rats weighing 200-250 g each (about 100-125 μ g/kg) twice daily for 3 days. Type B solutions (0.5 ml) were given to similar rats (2 - 2.5 mg/kg) by intraperitoneal injection, twice daily for three days. Control rats received the same solutions without the Reversins. During the three days of 25 injections and in a two-week follow-up period, no acute or subacute toxicity of the compounds was observed.

Additional toxicity testing can be performed by the methods of Pastan et al. (*Proc. Natl. Acad. Sci. USA* 85:4486-4490, 1988) and Tsuruo et al. (*Cancer Res.* 30 43:2905-2910, 1983).

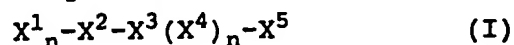
Other embodiments are within the claims set forth below.

What is claimed is:

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CLAIMS

1. A composition of the formula (I):



wherein

- 5 n is 0 or 1, and each n is the same or different;
 X^1 is selected from the group consisting of
 tert.-butyloxycarbonyl, BOC-Asu, Z-Asu,
 benzyloxycarbonyl, Glu(OBzl)-OBzl, Trp-OMe, Trp-
 Phe-OMe, Phe-Trp-OMe, Phe-Phe-OtBu, Trp-Trp-OtBu,
 10 indoleacetyl, benzoyl, alkylamine of 1-4 carbons,
 dibenzylamide, tryptamide, 1-amino-adamantine,
 aminomethylcyclohexane, indoline, phenylethylamide
 and dicyclohexylamide;
- X^2 is selected from the group consisting of Glu(OBzl),
 15 Asp(OBzl), succinyl, 0,0-dibenzoyltartaroyl,
 diphenoyl, muconyl, Thx, Cap, Asu, Nal, Pen, Phg,
 Dbt, Lys(BOC), Lys(Z), Cys(Bzl), Thr(Bzl)
 Glu(OtBu), tert.-Leu, Leu, Nle, Pro, Phe,
 Tyr(Bzl), and Ser(Bzl);
- 20 X^3 is selected from the group consisting of Asp, Asu,
 Lys, Glu, Trp, Thx, Cpa, Nal, Pen, Phg, Dbt,
 Glu(OtBu), tert.-Leu, Leu, Nle, Pro, Tyr, Phe, and
 Tyr(Bzl);
- X^4 is selected from the group consisting of BOC-
 25 Glu(OBzl), Glu(OBzl), OBzl, Bzl, BOC, BOC-
 Lys(BOC), Asu, Z-Glu(OtBu), Asp(OBzl), Asp(OBz)-
 OBzl, benzyloxycarbonyl, 0-(cyclo-hexyl),
 fluorenylmethyl ester, Glu(OtBu), Glu(OtBu)-OBzl,
 1-amino-adamantine, aminomethylcyclohexane,
 30 indoline, phenylethylamide, and dicyclohexylamide;
 and
- X^5 is selected from the group consisting of OMe, OBzl,
 OtBu, Phe-OMe, Trp-OMe, -O-(cyclohexyl),
 (chlorophenyl)-isobutylamide, fluorenylmethyl
 35 ester, ONp, 1-aminoadamantane,

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aminomethylcyclohexane, indoline,
phenylethylamide, and dicyclohexylamide.

2. The composition of claim 1, wherein said
formula (I) is BOC-Asp(OBzl)-Lys(Z)-OtBu.

5 3. The composition of claim 1, wherein said
formula (I) is succinyl-bis[Glu(OBzl)-OBzl].

4. The composition of claim 1, wherein said
formula (I) is Z-D-Glu(OBzl)-D-Asp(OBzl)-OBzl.

10 5. The composition of claim 1, wherein said
formula (I) is DBTA-bis(D-Phe-Trp-OMe).

6. The composition of claim 1, wherein said
formula (I) is DBTA-[Glu(OBzl)₂]₂.

7. The composition of claim 1, wherein said
formula (I) is N^α,N^ε-bis[BOC-Glu(OBzl)]-Lys-OMe.

15 8. The composition of claim 1, wherein said
formula (I) is BOC-Tyr(Bzl)-Tyr(Bzl)-OMe.

9. The composition of claim 1, wherein said
formula (I) is BOC-D-Ser(Bzl)-Lys(Z)-OtBu.

20 10. The composition of claim 1, wherein said
formula (I) is BOC-Glu(OBzl)-Lys(Z)-OtBu.

11. The composition of claim 1, wherein said
formula (I) is BOC-Glu(OBzl)-Lys(Z)-OMe.

12. The composition of claim 1, wherein said
formula (I) is N^α,N^ε-bis[BOC-Lys(BOC)]Lys-OMe.

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13. The composition of claim 1, wherein said formula (I) is N^α, N^ϵ -bis[Z-Glu(OtBu)]Lys-OMe.

14. The composition of claim 1, wherein said formula (I) is combined with a pharmaceutically
5 acceptable carrier.

15. The composition of claim 1, wherein said formula (I) is combined with a drug.

16. A method for preparing the composition of claim 1, said method comprising the steps of:

- 10 (a) providing a combination of one or more of said X^1_n , X^2 , X^3 , X^4_n , X^5 , or $X^1_n X^2$ in a solution;
(b) cooling said solution;
(c) adjusting the pH of said solution to the neutral range; and
15 (d) purifying said formula (I) from said solution.

17. The method of claim 16, wherein said method comprises active ester coupling.

18. The method of claim 16, wherein said method
20 comprises a dicyclohexylcarbodiimide condensation.

19. A method of reducing the activity of the multidrug transporter protein in a mammal, said method comprising administering to said mammal an amount of the composition of claim 1 that is effective for reducing
25 said activity.

20. The method of claim 19, wherein said method is used to lower resistance to a drug.

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21. The method of claim 20, wherein said drug is selected from the group consisting of a chemotherapeutic drug, an antiparasitic drug, and an antibiotic drug.

22. The method of claim 19, wherein said method
5 is used to facilitate administration of a drug through the blood-brain barrier or through the blood-testis barrier.

23. The method of claim 19, wherein said composition is co-administered with a drug.

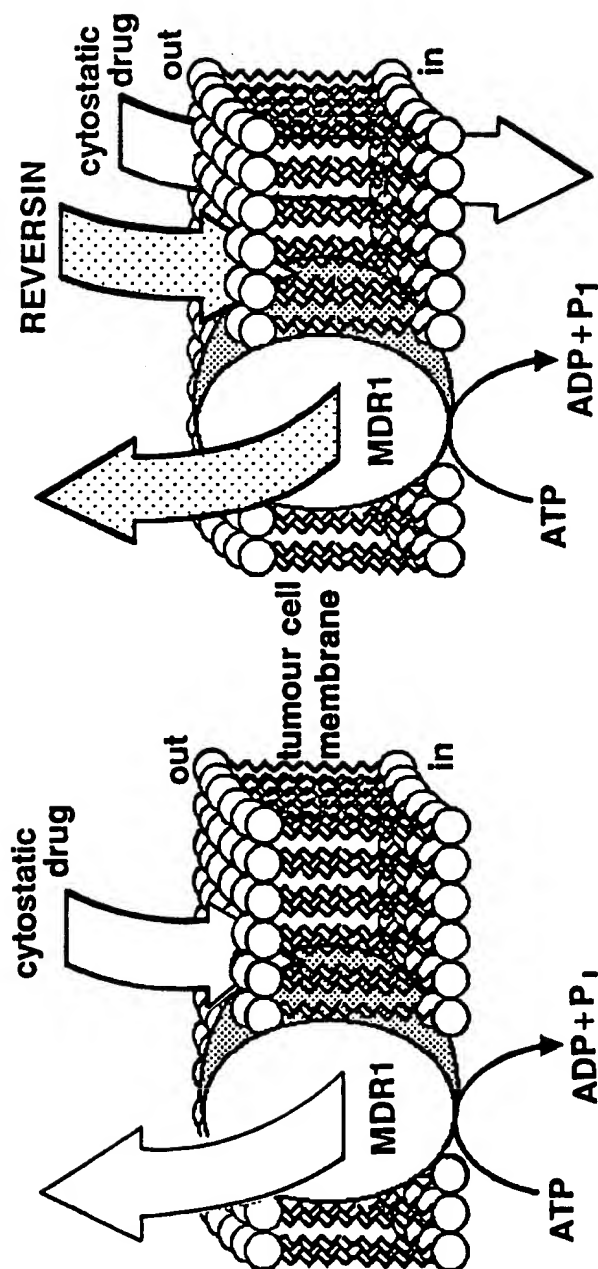
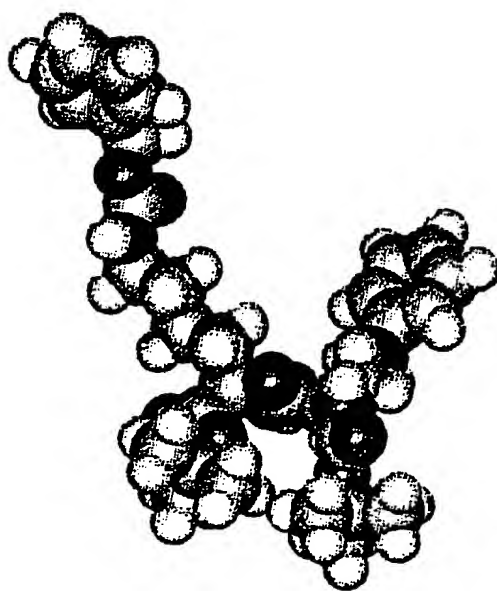


FIG. 1

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BOC-Asp(Obzl)-Lys(Z)-OtBu

FIG. 2

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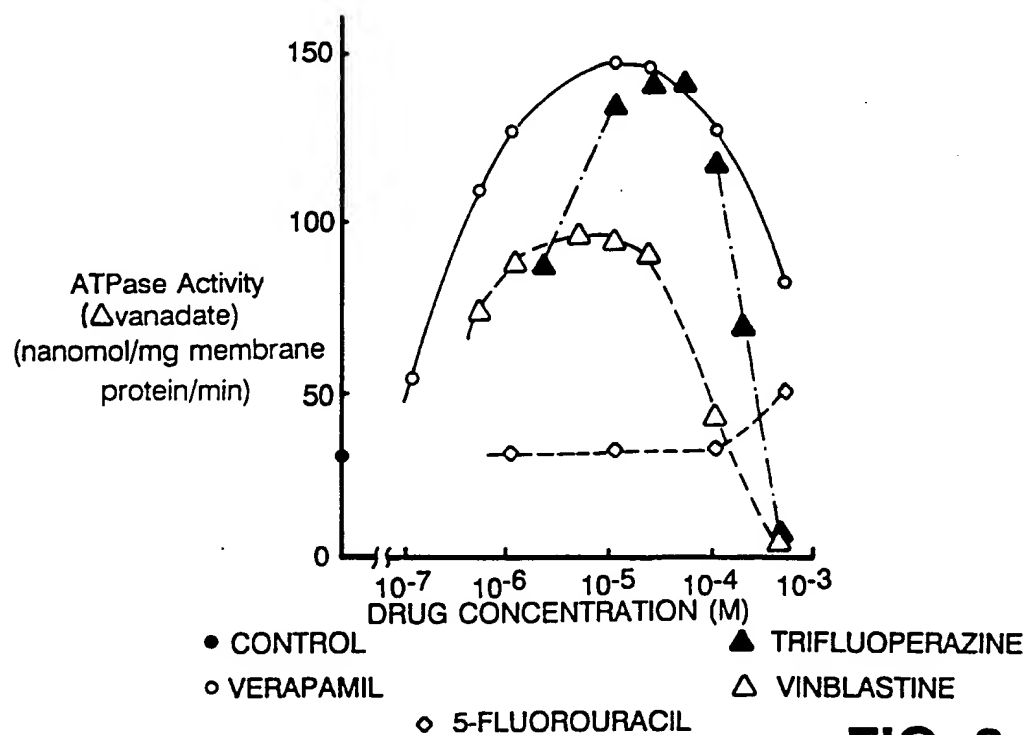


FIG. 3

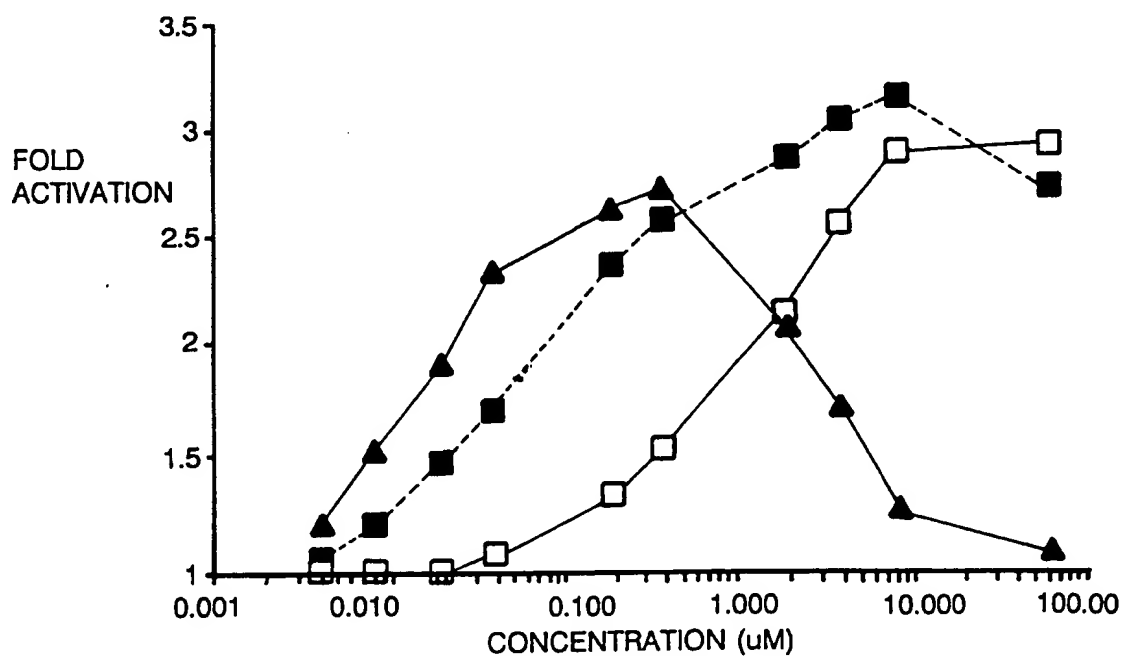
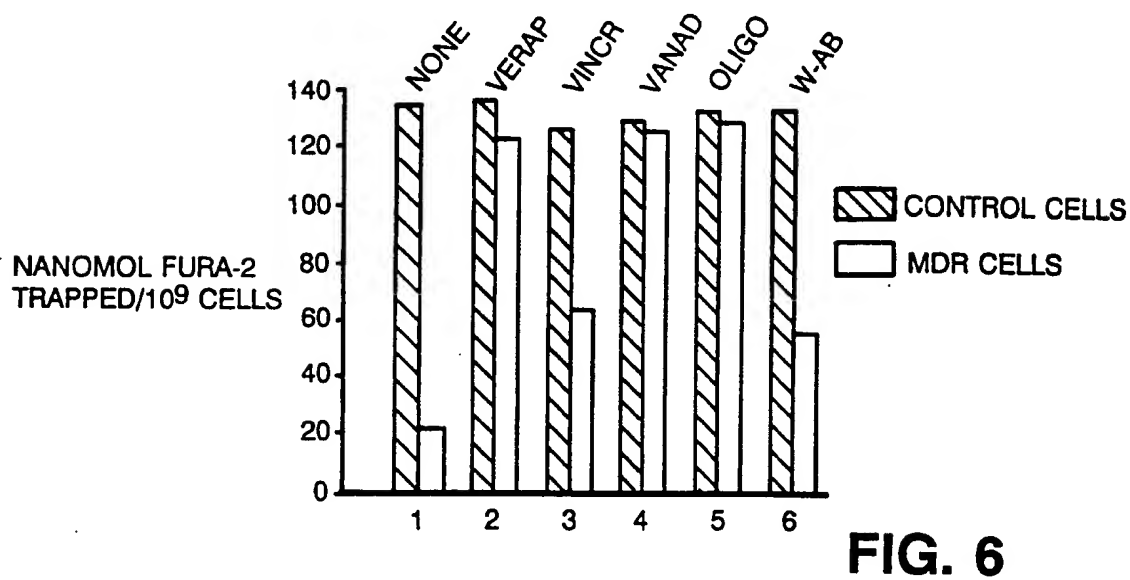
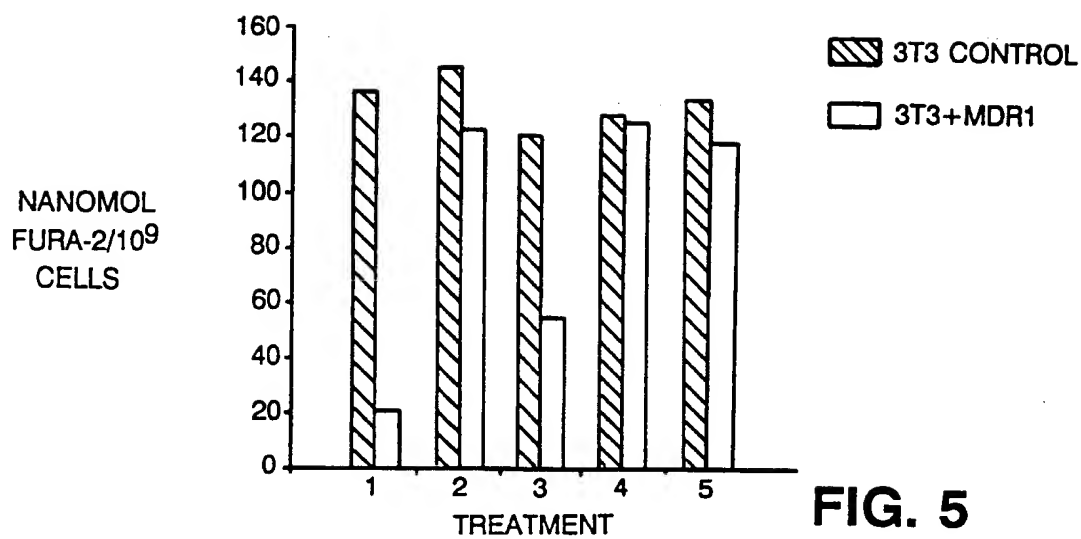
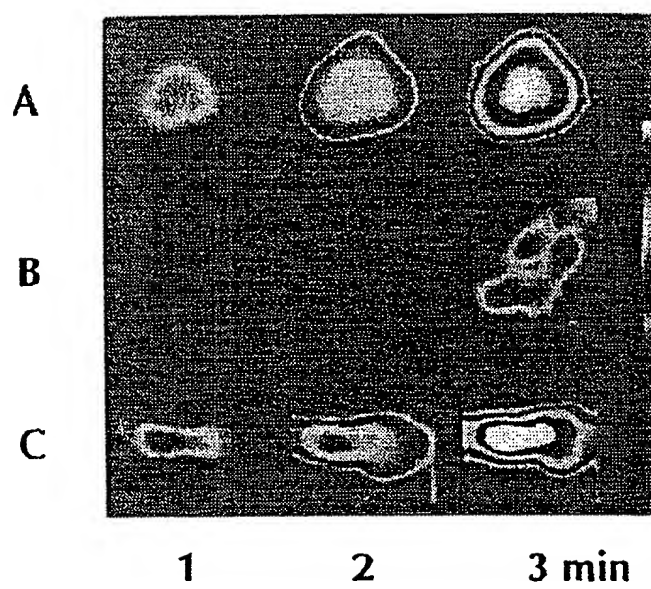


FIG. 4

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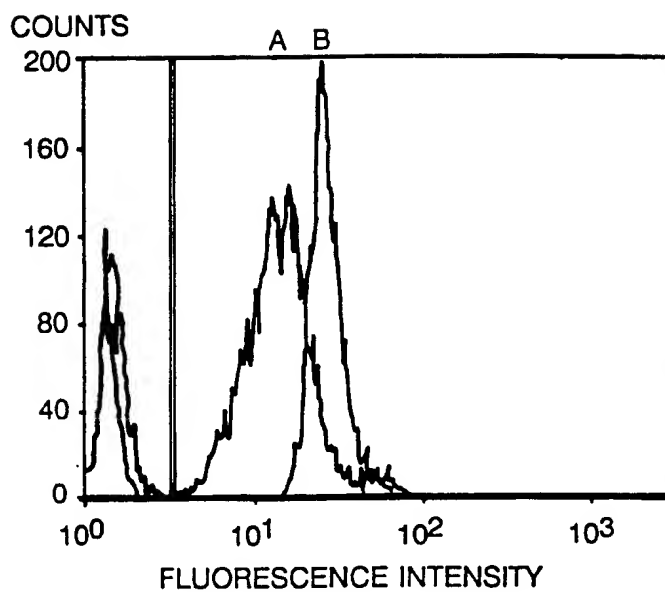
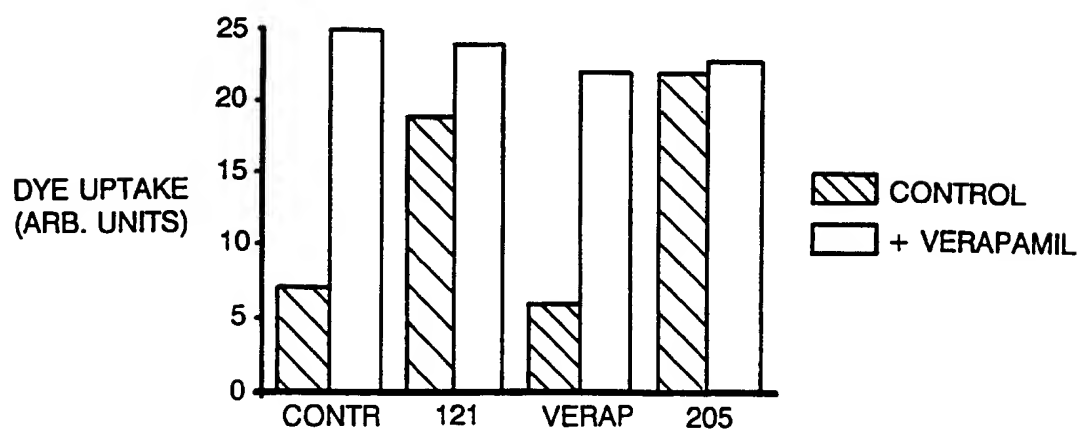


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**FIG. 7**

RECTIFIED SHEET

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**FIG. 8****FIG. 9**

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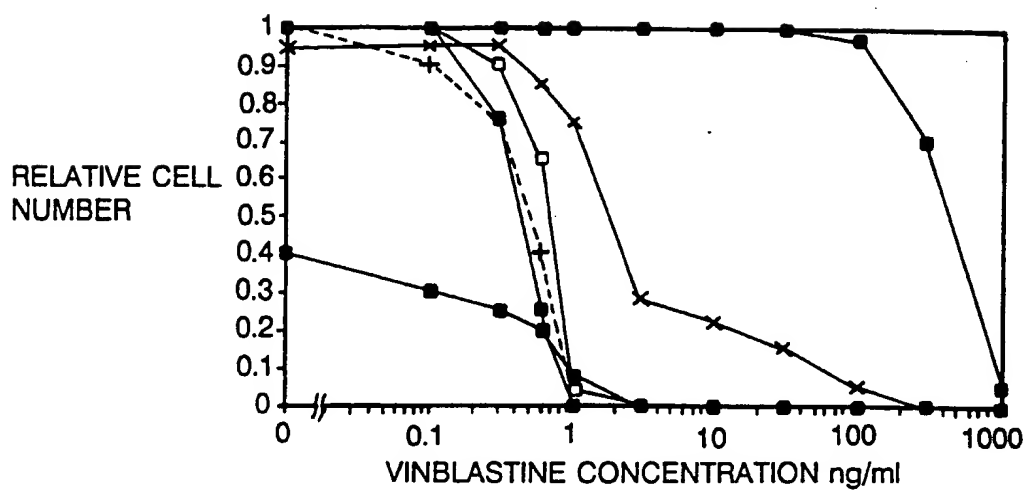


FIG. 10A

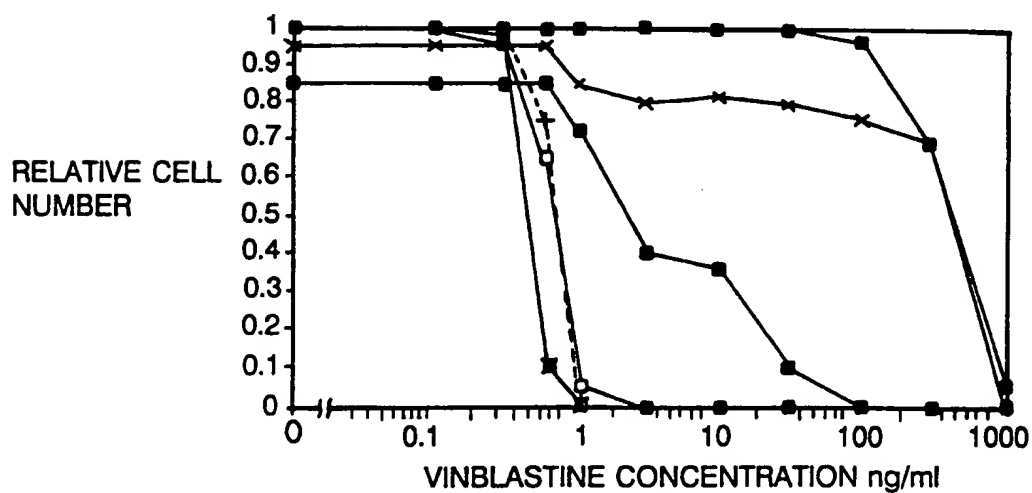


FIG. 10B

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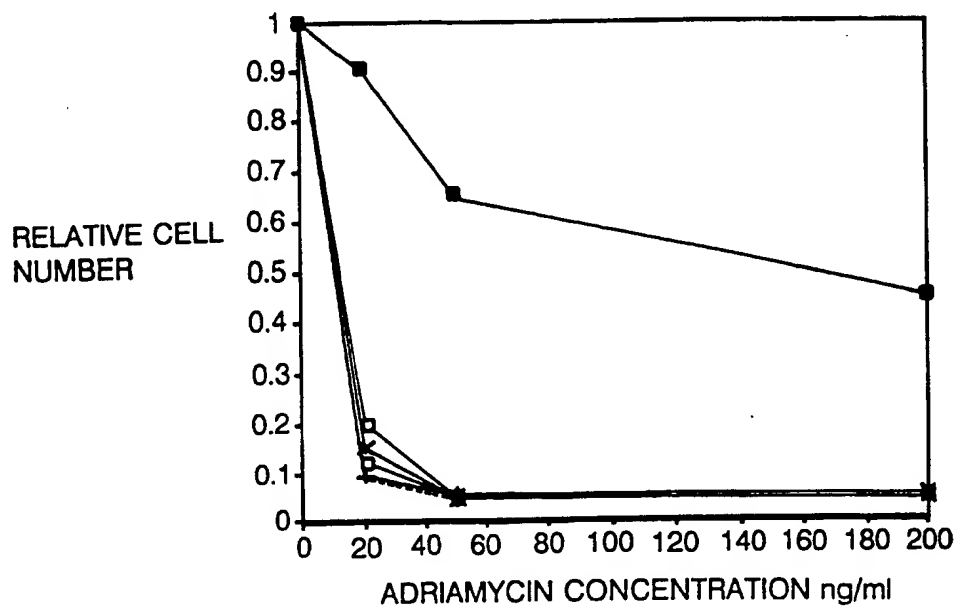


FIG. 11A

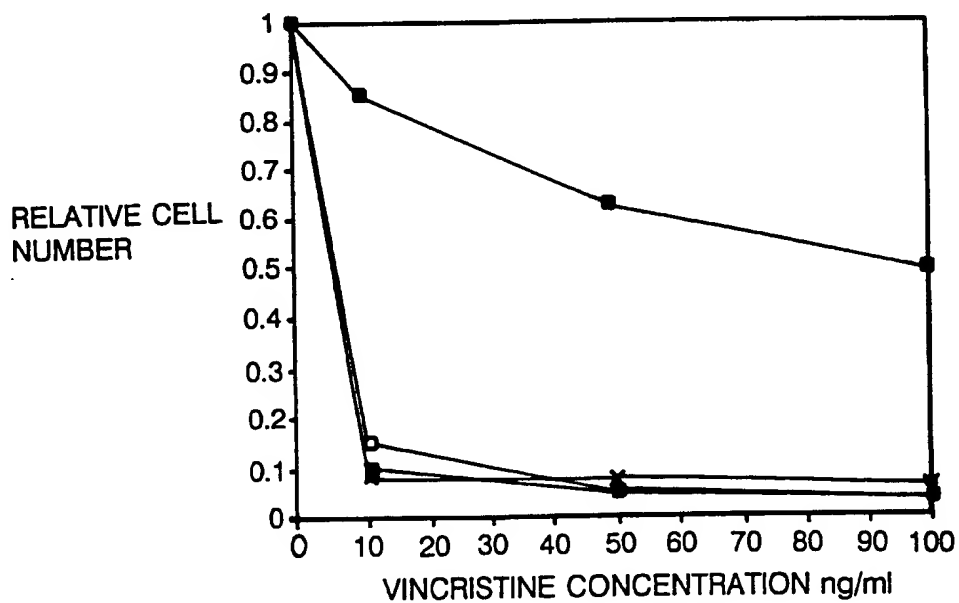


FIG. 11B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 94/00144

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 07 K 4/00, A 61 K 38/03

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 07 K, A 61 K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SU, A, 1 544 778 (HETEROORG CPDS AG USSR)	1
A	23 February 1990 (23.02.90), abstract.	8, 16
X	US, A, 4 028 319 (JONES et al.) 07 June 1977 (07.06.77), example 12; column 3, lines 15-19.	1, 14

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 January 1995 (11.01.95)

Date of mailing of the international search report

31 January 1995 (31.01.95)

Name and mailing address of the ISA/ AT

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Telephone No.

1/53424/226

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 94/00144

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 19-23
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 19-23 are considered to be a method for treatment of the human or animal body by therapy, which is subject matter the International Searching Authority is not required to search, all claims have been searched completely. Relevant documents are cited within the search report.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB 94/00144

In Recherchenbericht angeführtes Patentedokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
SU A 1544778		keine - none - rien	
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		AU B2 508251	13-03-80
		BE A1 854397	09-11-77
		DE A1 2720152	17-11-77
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		ES A5 458537	17-04-78
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		FR B1 2397394	25-04-80
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		NZ A 184020	11-12-79
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		ZA A 7702758	28-06-78

